

Art Unit: 1652

Application No.: 09/424,686

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING  
NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

- ☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.
- ☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a Sequence Listing as required by 37 C.F.R. 1.821(c).
- ☐ 3. A copy of the Sequence Listing in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).
- ☒ 4. A copy of the Sequence Listing in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up Raw Sequence Listing.
- ☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).
- ☐ 6. The paper copy of the Sequence Listing is not the same as the computer readable form of the Sequence Listing as required by 37 C.F.R. 1.821(e).
- ☒ 7. Other:
- Applicant Must Provide:**
- ☒ An initial or substitute computer readable form (CRF) copy of the Sequence Listing.
- ☒ An initial or substitute paper copy of the Sequence Listing, as well as an amendment directing its entry into the specification.
- ☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:  
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For Patent software help, call (703) 308-6856

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mixture (1 minute 94°C, 2 minutes at 60°C, 3 minutes at 72°C) with the hTC cDNA in pT7T3D and primers 1 (5' GAGTGTGTACGTCGTCGAGCTGCTCAGGTC 3') (SEQ ID NO: 13) and 4 (5' CACCCTCGAGGTGAGACGCTCGGCC 3') (SEQ ID NO: 14) [lane 4] and, especially, with primers 6 (5' CTCGTAGTTGAGCACGCTGAACAGTG 3') (SEQ ID NO: 15) and 7 (5' GCCAAGTTCCTGCACTGGCTGATGAG 3') (SEQ ID NO: 16) [lane 5] was applied to lanes 4 and 5.

Fig. 4: Detail from a comparison of the protein sequences of the *Euplotes* p123 (p123) and human (phTC) catalytic telomerase subunits.

The conditions (ktuple, gap penalty and gap length penalty) are listed for the Lipman-Pearson protein comparison, using the Lasergene program software (Dnastar, Inc.), which is depicted in this figure. The amino acid residues are depicted in accordance with their single-letter code. The amino acids which are identical between *Euplotes aediculatus* p123 and the identified EST,, are also highlighted using the corresponding letter from the single-letter code. Amino acids which are not identical but whose function is similar or comparable are marked by a:.

Fig. 5: Part of a comparison of the protein sequences of the catalytic telomerase subunits of *Euplotes* p123 (p123), and yeast (est2p).

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Fig. 6: Detail from a comparison of the protein sequences of the yeast (est2p) and human (phTC) catalytic telomerase subunits.

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### **Example 6**

For cloning the 5' end of the hTC-cDNA, three consecutive RACE (rapid amplification of cDNA ends) reactions were carried out in addition to the homology screening described in Example 8. Marathon-Ready cDNA (Clontech) from the human leukaemia cell line K562 or from human testis tissue was employed as the cDNA source. The implementation of the individual RACE rounds, as well as the results obtained, are described below.

In addition to this, the sequence information obtained in the RACE rounds was used in order to amplify the individual fragments from a contiguous cDNA clone by means of PCR.

#### **RACE round 1:**

In a final volume of 50 µl, 10 pmol of dNTP-mix were added to 5 µl of K562 MarathonReady cDNA (from Clontech, Catalogue Number 7441-1), and a PCR reaction was carried out in 1 x Klen Taq PCR reaction buffer and 1 x advantage Klen Taq polymerase mix (from Clontec). 10 pmol of the internal gene-specific primer GSP2 (5' -

GCAACTTGCTCCAGACACTTCTTCCGG-3') (SEQ ID NO: 17) from the 5' region of the hTC-EST clone and 10 pmol of the Marathon Adaptor primer API (5' -

CCATCCTAATACGACTCACTATAGGGC-3'; from Clontech) (SEQ ID NO: 18) were added as primers. The PCR was carried out in 4 steps. After a one-minute denaturation at 94°C, denaturation was then carried out for 5 cycles of 30 sec at 94°C and the primers were then subsequently annealed for 4 min at 72°C and the DNA chain was extended. There then followed 5 cycles in

which the DNA was denatured for 30 sec at 94°C but the subsequent primer extension took place for 4 min at 70°C. Finally, 22 cycles were then carried out in which, after the 30 sec DNA denaturation, the primer annealing and chain extension took place for 4 min at 68°C.

Following this PCR, the PCR product was diluted 1:50. 5 µl of this dilution were used in a second "nested" PCR together with 10 pmol of dNTP-mix in 1 x 10 Klen Taq PCR reaction buffer and 1 x Advantage Men Taq polymerise mix and also 10 pmol of primer GSP2 and

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10 pmol of the "nested" Marathon Adaptor primer AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3'; from Clontech) (SEQ ID NO: 19). The PCR conditions corresponded to the parameters selected in the first PCR. As the only exception, only 16 cycles were chosen, instead of 22 cycles, in the last PCR step.

A DNA fragment of 1153 bp in length was obtained as the product of this nested RACE PCR. This fragment was cloned into the TA cloning vector pCR2.1 from Invitrogen and subjected to complete double-strand sequencing (Fig. 8 and SEQ ID No. 3).

Nucleotides 974 to 1153 represent the nucleotide region 1629 to 1808 of the hTC-cDNA which is depicted in Fig. 1. The nucleotide region extending from bp 1 to bp 973, which does not exhibit any homology with the hTC-cDNA sequence shown in Fig. 1, represents intron sequences of the hTC gene (data not shown). A 3' splice consensus sequence is located at the exon-intron transition. The presence of intron sequences could be due to using incompletely spliced mRNA as the starting substance for the cDNA synthesis. Genomic DNA contamination in the cDNA could also be an explanation for intron sequences being found.

#### RACE round 2:

Based on the sequence data obtained in the first RACE round, a second RACE was carried out using the gene-specific primer GSP5 from the 5' region of RACE product 1 (5'-GGCAGTGACCAGGAGGCAACGAGAGG-3') (SEQ ID NO: 20) and the API primer. Marathon-Ready cDNA from human testis (from Clontech; Catalogue Number 7414-1) was used as the cDNA source. The same PCR conditions were selected as in the 1st PCR in RACE round 1. The 1st PCR was also followed, in RACE round 2, by a 2nd "nested" PCR using diluted PCR product as the cDNA source. The gene-specific primer GSP6 from the 5' region of RACE product 1 (5'-GGCACACTCGGCAGGAAACGCACATGG-3') (SEQ ID NO: 21) and the AP2 primer were used as the "nested" PCR primers. The conditions corresponded to parameters for the nested PCR from RACE round 1.

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The PCR product of 412 by in length from the nested PCR of RACE round 2 was cloned into the TA cloning vector pCRII-Topo from Invitrogen and sequence completely (Fig. 9 and SEQ ID No. 4). The sequence segment from by 267 to by 412 is completely homologous with the 5' region of the product from RACE 1. The region from by 1 to by 266 extends RACE product 1 at the 5' end. This RACE product 2 is probably, in its entirety, an intron region of the hTC gene (data not shown).

### RACE round 3:

A third RACE round led to the identification of hTC-cDNA regions which were located further on in the 5' direction. Using the sequence results from RACE round 2 as a base, a gene-specific primer GSP9 (5'-CCTCCTCTGTTCACCTGCTCTGGCC-3') (SEQ ID NO: 22) was selected from the 5' region of RACE product 2 and used in a new RACE together with the API primer and Marathon-Ready cDNA from human testis (from Clontech). The RACE conditions were the same as those used in the 1st PCR in RACEs 1 and 2. In the "nested" RACE which followed, and which took place, in accordance with the "nested" RACEs in rounds 1 and 2, using the gene-specific primer GSP10 from the 5' region of RACE product 2 (5'-CGTAAGTTTATGCAAACCTGGACAGG-3') (SEQ ID NO: 23) and AP2, a fragment of 1012 by in length (Fig. 10 and SEQ ID No. 5) was amplified and cloned into the TA cloning vector pCRII-TOPO. Subsequent sequencing showed that the 3' region of this RACE fragment (bp 817 - bp 1012) evidently still constitutes an intron sequence of the hTC gene. The region from by 889 to by 1012 is completely homologous with the 5' region of RACE product 2. On the other hand, the 5' region of this fragment, from by 1 to by 816, is identical to the by 814 - bp 1629 region of the hTC-cDNA which is shown in Fig. 1. A potential 5' splice consensus sequence is located at the exon-intron transition.

### Example 7

A PCR was carried out in order to clone a contiguous fragment from the sequence information obtained from RACE 2 and clone AA281296. Marathon-Ready cDNA from human testis (from Clontech; Catalogue Number 7414-1) was used as the cDNA source. The



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PCR mixture was as described under RACE 1 (compare Example 6) but using the primers C5F (5'-CGAGTGGACACGGTGATCTCTGCC-3') (SEQ ID NO: 24) from the 5' region of RACE 2 and primer C3B (5'-GCACACCTTTGGTCACTCCAAATTCC-3') (SEQ ID NO: 25) from a 3' region of clone AA281296. The PCR was carried out in 2 steps. After a one-minute denaturation at 94°C, denaturation was then carried out for 36 cycles of 30 sec at 94°C and, after that, the primers were annealed, and the DNA chain was extended, for 4 min at 68°C.

A DNA fragment of 2486 by in length, which is designated the C5F fragment below, was obtained as the product of this PCR. This fragment was cloned into the TA cloning vector pCRII-TOPO from Invitrogen and subjected to complete double-strand sequencing. A comparison of the DNA sequences of the C5F fragment and the AA281296 clone showed that there was an in-frame insertion of 182 by between RT motif 3 and RT motif 4 (Positions 2352 to 2533, Fig. 1). A further comparison of DNA of the C5F fragment with the sequences from the three RACE rounds made it clear that an intron which was already identified in RACE 2 was present at the 3' end of C5F. A 3' splice consensus sequence is located at the exon-intron transition. In summary, the DNA sequence of the C5F fragment is consequently composed of the sequence information shown in Fig. 9 (Position 64 to 278) and the sequence data shown in Fig. 1 (Positions 1636 to 3908).

### **Example 8**

For cloning the 5' end of the hTC-cDNA, a homology screening (Ausubel *et al.*, 1987) was carried out in addition to the RACE protocol described in Example 6. A human erythroleukaemia 5'-stretch plus cDNA library (from Clontech, cat. No. HL5016b) from the human leukaemia cell line K562 was used as the cDNA source. Approximately  $3 \times 10^6$  Pfu of this random and oligo-dT-primed library were plated out and used for screening as described in Ausubel *et al.* (1987). A radioactively labelled hTC-DNA fragment of 719 by in length (Positions 1685 to 2404, corresponding to Fig. 1) was used as the probe.

Following a rescreening with the same hTC probe, the X clone 12 was verified as being positive out of 20 putatively positive X clones. Following plaque purification and ? DNA

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lymphoma) tested (Fig. 15, Fig. A). In the comparison, the hTC mRNA was expressed most strongly in the leukaemia cell lines K-562 and HL-60 (Fig. 15, Fig. A). By contrast, it was not possible to detect the hTC transcript in the normal tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) which were tested (Fig. 15, Fig. B). This observation is not surprising since it was not possible to detect any telomerase activity, either, in these tissues (Kim *et al.*, 1994).

These data indicate that the induction of hTC expression plays an important role in activating the telomerase during tumour development.

### **Example 11**

Several PCR products, whose sizes only differed from each other to a minimal extent, were always obtained when the hTC cDNA fragments from various cDNA libraries (Clontech Marathon Ready cDNA from the human leukaemia cell line K562 and from human testis and also cDNA from the human promyeloid leukaemia cell line HL60) were subjected to PCR amplification. In order to elucidate the differences between the different hTC-PCR products, a fragment of the hTC cDNA depicted in Fig. 1 extending from by 1783 to by 3901 was amplified using the primers C5A (5'-CCGGAAGAGTGTCTGGAGCAAGTTGC-3') (SEQ ID NO: 26) and C3B (5'-GCACACCTTTGGTCACTCCAAATTCC-3') (SEQ ID NO: 25). Marathon-Ready cDNA from K562 leukaemia cells (from Clontech; Catalogue Number 7441-1) was used as the cDNA source (PCR1 and 2). In a third PCR, a hTC fragment, from by 1695 to by 3463, of the hTC cDNA in Fig. 1 was amplified from HL60 cDNA using the primers GSPI front (5'-GGCTGATGAGTGTGTACGTCGTCGAG-3') (SEQ ID NO: 27) and HTRT3A (5'-GGGTGGCCATCAGTCCAGGATGG-3') (SEQ ID NO: 28).

The conditions of the 3 PCR reactions are described below:

In the first PCR, and in a final volume of 50 µl, 10 pmol of dNTP mix were added to 5 µl of K562 Marathon-Ready cDNA, and a PCR reaction was carried out in 1 x Men Taq PCR reaction buffer and 1 x Advantage Klen Taq polymerase mix (from Clontech). 10 pmol of

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software package, DNASTAR Inc., Madison, WI, USA), two peptides were chosen which, with a certain degree of probability, evoke an immune response. These are the following peptides, which are depicted in the one-letter code for amino acids:

- B: C-K-R-V-Q-L-R-E-L-S-E-A-E-V-R-Q - CONH<sub>2</sub>/Pos. 594 - 608 (SEQ ID NO: 31)  
C: C-Q-E-T-S-P-L-R-D-A-V-V-I-E-Q-S-S-S-L-N-E - CONH<sub>2</sub>/Pos. 781-800 (SEQ ID NO: 32)

The cysteines which are underlined are not derived from the telomerase sequence but were additionally added on as linkers for the coupling.

The peptides were coupled to keyhole limpet hemocyanin (KLH) using the thiol-reactive coupling reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). Two rabbits were in each case immunized with these coupled peptides at intervals of from 2 to 4 weeks. Prior to immunization, 5 ml of blood were withdrawn in order to obtain preimmune sera. After 4 immunizations, 5 ml of blood were likewise withdrawn for obtaining immune sera. These sera were tested for reactivity with fusion proteins (Example 13) in a Western blotting experiment (Ausubel *et al.*, 1987).

### **Example 13**

Bacterial expression experiments were carried out in order to be able to analyse the protein of the catalytic telomerase subunit.

The constructs of these experiments are described below:

For the expression construct pMaIEST, the insert in the AA281296 clone mentioned in Example 2 was excised with restriction enzymes Eco RI and Not I and the cleavage sites were filled in using the Klenow fragment (Ausubel *et al.*, 1987); the insert was then cloned into the given reading frame of the maltose-binding protein of the bacterial expression vector pMAL-C2 (from New England Biolabs). Vector pMAL-C2 was digested with restriction

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enzyme Pst I and the protruding single-strand ends were removed with T4 DNA polymerase (Ausubel *et al.*, 1987).

The expression construct pMalA1 contains the nucleotide sequence of Fig. 1 from Position 1789 to Position 3908. This DNA fragment was amplified from a commercially available K562 Marathon-Ready cDNA library (from Clontech, Catalogue Number 7441-1) by means of PCR using the primers C5A (5'-ACCGGAAGAGTGTCTGGAGCAAGTTG-3') (SEQ ID NO: 26) and C3B (5'-GCACACCTTTGGTCACTCCAAATTCC-3') (SEQ ID NO: 25), and cloned into the TA cloning vector pCRII-TOPO from Invitrogen. The PCR conditions were as described in Example 7. For the expression construct pMalA1, the insert was excised using the restriction enzyme Eco RI and the cleavage sites were filled in using the Klenow fragment (Ausubel *et al.*, 1987); the insert was then cloned into the bacterial expression vector pMAL-C2 (from New England Biolabs) which had been cleaved with the restriction enzyme Xmn I.

These constructs were then used for protein expression in the bacterial strain *E. coli* DH5a. The expression conditions were those as described in the instructions provided by New England Biolabs (Catalogue Number 800). The bacterial lysates which were prepared were tested in a Western blotting experiment (Ausubel *et al.*, 1987).

#### **Example 14**

The bacterial lysates from Example 13 were analysed in a Western blot (Ausubel *et al.*, 1987) using the antisera from Example 12.

Since the proportion of the fusion represented by the maltose-binding protein is about 43 kDa in size, fusion proteins of about 74 kDa and 106 kDa are expected for the pMalEST and pMalA1 constructs, respectively.

When comparing the preimmune sera with the sera following the first immunization, it becomes evident that specific antibodies were formed against the B and C epitopes (Fig. 16). Furthermore, in addition to the expected 74 kDa and 106 kDa proteins, respectively, smaller



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protein fragments were also observed which react with the antisera. These smaller products probably originate from premature products.

Only the epitope for serum B is present on the fusion protein from the expression using pMaIEST. By contrast, the epitopes for sera B and C are present on the fusion protein from pMalA1. For this reason, antiserum C does not recognize the pMaIEST expression product and only recognizes the larger protein fragments from the expression experiments using pMalA1. This observation underlines the high degree of specificity of the antisera which were generated.

### **Example 15**

In order to be able to analyse the protein of the catalytic telomerase subunit, the protein component should be reconstituted *in vitro* together with the RNA component.

The constructs for these experiments are described below:

The RNA component of 504 nt in length (Feng *et al.*, 1995) was amplified from a 293 cell cDNA library using the primers HTR9BAM (5'-CGCGGATCCTAATACGACTCACTATAGGGTTGCGGAGGGTGGGCCTG-3') (SEQ ID NO: 29) and HTR2BAM (5'-CGCGGATCCCGGCGAGGGGTGACGGATGC-3) (SEQ ID NO: 30). Primer HTR9BAM contains a T7 promoter from nucleotide 10 to 29. In the PCR, 10 pmol of dNTP mix were added, in a final volume of 100 µl, to 3 µl of cDNA from 293 cells, and a PCR reaction was carried out in 1 x PCR reaction buffer containing 0.5 µl of Taq polymerase (from Gibco). 10 pmol of each of the primers HTR9BAM and HTR2BAM were added. The PCR was carried out in 3 steps. A ten-minute denaturation at 94°C was followed by 35 PCR cycles in which the DNA was first of all denatured for one minute at 94°C and, after that, the primers were annealed, and the DNA chain was extended, for 2 min at 62°C. In conclusion, there followed a chain extension for 4 min at 72°C. The resulting PCR products were cloned, after a restriction digestion with Bam HI, into the Bam HI cleavage site of vector pUC19 in such a way that the

protein fragments were also observed which react with the antisera. These smaller products probably originate from premature products.

Only the epitope for serum B is present on the fusion protein from the expression using pMaIEST. By contrast, the epitopes for sera B and C are present on the fusion protein from pMalA1. For this reason, antiserum C does not recognize the pMaIEST expression product and only recognizes the larger protein fragments from the expression experiments using pMalA1. This observation underlines the high degree of specificity of the antisera which were generated.

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